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Compartmentalisation of BMP and BMP antagonists in lymphoid progenitors and supporting microenvironments and functional implications

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Abstract

Bone Morphogenetic protein (BMP) signaling regulates lymphopoiesis in bone marrow and thymus via the interaction of hemato-lymphoid progenitors with the stroma microenvironment. Despite increasing functional evidence for the role of Bmp signaling in lymphopoiesis, little is known on the spatial distribution of Bmp/Bmp antagonists in the thymus and how Bmp signals exerts specific functions in developing lymphocytes. We analyzed expression of Bmp/Bmp antagonists in the thymus and bone marrow and determined the topology of Bmp/Bmp antagonist expression using lacZ reporter mice. *Bmp4*, *Bmp7*, *Gremlin*, and *Twisted gastrulation* (*Twsg1)* are all expressed in the thymus and expression was clearly different for each gene investigated. Expression was seen both in cortical and medullary regions suggesting that Bmp signals regulate all stages of T-cell development. Two genes in particular, *Bmp7* and *Twsg1*, were dynamically expressed in developing T- and B-lymphocytes. Their conditional ablation in all hematopoietic cells surprisingly did not affect the steady state of B- and T-cell development. This indicates that both lymphoid cell-derived BMP7 and TWSG1 are dispensable for normal lymphopoiesis and that bone-marrow stroma derived TWSG1 is responsible for the lymphoid defects observed in *Twsg1* null mice. In summary our data demonstrate a complex network of lymphoid and stroma derived Bmp signals involved in the orchestration of lymphopoiesis in both bone marrow and thymus.

Introduction

Both T- and B-lymphocytes originate from a common lymphoid precursor but their development requires distinct microenvironments. B-lymphocytes develop in the bone marrow whereas T-cells mature in the thymus. It is well established that reciprocal interactions between developing lymphocytes and stroma cells from the respective organs are key to their successful maturation [1, 2]. Several growth and differentiation factors have been identified in stromal cells that are instrumental for supporting T-cell and B-cell development [3, 4]. However, less is known about signals that originate from the developing lymphocytes to reciprocally support stromal cell function [5].

Embryonic development and adult homeostasis of most tissues are orchestrated to a large degree by only a few families of conserved signaling factors: the Hedgehog (Hh)-, Notch-, Wnt/ß-catenin-, Tumor-Growth-Factor (TGF)/Bone Morphogenetic Protein (BMP)-, and Fibroblast Growth Factor (FGF) families. All these signaling pathways are also important for the development of the lymphoid organs and lymphocytes themselves [6-9].

Bmps, which belong to the TGFβ superfamily, are evolutionary highly conserved molecules and can be separated into two subgroups. Bmp2 and Bmp4 are orthologues of *Drosophila* Decapentaplegic (Dpp), whereas Bmp5, Bmp6, and Bmp7 belong to the Glass-bottom-boat (Gbb) subgroup. The name Bmp originates from the molecule's ability to induce ectopic bone formation [10]. Bmps are secreted signaling molecules and, contrary to their name, have critical functions in many different biological processes outside of bone formation. They are involved in many aspects of embryonic development [11, 12], homeostasis and repair of various tissues [13] including the hematopoietic system [14, 15].

Bmps signal via specific heterodimeric Bmp receptors consisting of a type I receptor (Alk1, Alk2, Alk3, Alk6) and a type II receptor (BmpRII, ActRII, ActRIIB) subunit. Receptor engagement leads to the phosphorylation of a type I receptor by a type II receptor, which in turn facilitates the activation of either the BMP-specific Smad1/5/8 signaling pathway [16] or non-specific signal transduction pathways such as MAPK/PI3K/Akt [17]. In Smad-dependent signaling, Smad1/5/8 are phosphorylated and translocated together with the co-Smad Smad4 to the nucleus to exert their cellular effects [18].

Receptor engagement by Bmps is highly regulated in the extracellular space by secreted Bmp antagonists such as Gremlin, Noggin, Chordin, and Twisted Gastrulation (TWSG1) that bind Bmps with high affinity and thus prevent receptor engagement [19]. The binding affinities of the various Bmps to antagonists as well as receptors differ [20]. Thus, the various Bmps and Bmp antagonists are not simply redundant but are required for the precise spatiotemporal regulation of Bmp signaling. By controlling Bmp expression as well as exploiting the different binding affinities to receptors and antagonists [20], a precise spatio-temporal regulation of Bmp signals is possible. Because of this complexity, the precise contribution of these different Bmp signals to complex biological processes is still poorly understood.

Bmp signaling plays a prominent role during hematopoiesis: it modulates the developmental program of human hematopoietic stem cells (HSC) [14, 15] and controls the size and number of the HSC niches [21]. Deregulation of Smad molecules affects normal hematopoietic growth and leads to neoplastic hematopoiesis [22].

Bmp signals also regulate thymopoiesis [6, 23, 24] and the development of thymocytes themselves [25-28]. Thymocyte development requires reciprocal interactions between thymic stroma and developing thymocytes [29]. Similarly, B-cell development requires

reciprocal interactions between developing B-lymphocytes and bone marrow stroma [5]. Previous studies have shown that thymic stroma derived Bmp2/4 inhibits T cell development [25-27]. Thymocytes can modulate the effect of Bmp2/4 by expressing the evolutionarily conserved Bmp modifier Twsg1 in a TCR-dependent manner [26, 30]. Known target genes of Smad-dependent signaling are the inhibitor of differentiation (Id) gene family [31]. Id proteins are important regulators of lymphocyte development [32] lending further evidence for an important role of Bmp signals in coordinating lymphocyte development.

In this study, we have mapped the site of expression for several Bmp and Bmp antagonists in the mouse thymus, as well as in subsets of developing T- and B-lymphocytes and examined the effects of conditional ablation of these two molecules in developing lymphocytes.

MATERIALS AND METHODS

Mice

Heterozygote *Bmp4*$^{+/lacZ}$ [33], *Bmp7*$^{+/lacZ}$ [34], *gremlin*$^{+/lacZ}$ [35], and *Twsg1*$^{+/lacZ}$ [36] reporter mice have been used in this study. Mice carrying conditional allele (*Twsg1*$^{fl/+}$) [37] were crossed with FlpCre mice to remove the neo cassette and were subsequently backcrossed to C57BL/6 mice for at least six to eight generations. The F1 progeny homozygous for the floxed allele (*Twsg1*$^{fl/fl}$) was crossed with *Twsg1*$^{wt/ko}$vav::iCre mice to generate mice with a conditional loss of *Twsg1* in the hematopoietic cell compartment (*Twsg1*$^{fl/KO}$*Vav::iCre*; designated as Twsg1cKO here). Mice carrying conditional allele (*Bmp7*$^{fl/+}$) [38] were backcrossed into C57Bl/6 mice for at least seven generations. The F1 progeny homozygous for the floxed allele (*Bmp7*$^{fl/fl}$) was crossed with *Bmp7*$^{wt/\Delta}$vav::iCre mice to generate mice with a conditional loss of *Bmp7* in the hematopoietic cell compartment (*Bmp7*$^{fl/\Delta}$*vav::iCre*; designated as Bmp7cKO here). Congenic C57Bl/6-CD45.1 mice were provided by A.Potocnik (NIMR, London, UK). Mice were maintained at the B.S.R.C. Al. Fleming animal facility under specific pathogen free conditions. Experiments on live animals were approved by the Hellenic Ministry of Rural Development (Directorate of Veterinary Services) and by BSRC Alexander Fleming's Animal Research and Ethics Committee for compliance to Federation of European Laboratory Animal Science Associations' regulations.

LacZ staining and immunohistochemistry

O.C.T. (BDH) embedded tissues were 'snap frozen' in liquid nitrogen steam. 6-7μm cryostat sections were placed in gelatin-coated slides, air-dried, fixed in cold gluteraldehyde/formaldehyde or PFA/acetone and incubated with 2mg/mL X-gal (HT

Biotechnology, Cambridge, UK) at 37°C overnight. Subsequent staining was performed with the following antibodies CD3 (KT3), CD25 (7D4), CD205 (DEC205)(NLDC)(all Biolegend, eBiosciences or BD Biosciences), Cytokeratin-8 (CK8)(Troma-1, DSHB, Iowa). Sections were developed using an appropriate ABC-HRP kit (Vector Laboratories, Burlingame, CA).

Flow cytometry and sorting

For surface antigens, staining was performed using standard procedures. The following antibodies were used CD4 (GK1.5, PECy5.5), CD8 (53-6.7, PE), Ly6C (AL-21, FITC), CD31 (390, biotin), B220 (RA3-6B2, PE), CD19 (6D5, APC), CD25 (PC61, Alexa-700), cKIT (2B7, PE-Cy7), FITC), CD45.1 (A20, APC-Cy7), CD45.2 (104, Alexa-700) (all Biolegend, eBioscience, or BD Biosciences), IgM (goat $F(ab')_2$ (Southern Biotech). For FDG detection, labeled cells were incubated under hypotonic conditions with 2mM FDG (Marker Gene Technologies) at 37°C for 1min. Aquistion was on FACS CantoII™ using FACSDIVA™ software (BD Biosciences). Analysis was performed using FlowJo™ (TreeStar). DN thymocytes were sorted as lineage negative ($CD4^-$, $CD8^-$, $CD11b^-$, $CD45RB^-$, $CD49b^-$, $TER119^-$) after depletion using the IMag mouse CD4 lymphocyte enrichment sets (BD Biosciences) followed by FACS sorting on a FACS Aria (BD Biosciences).

Repopulation assay

$1x10^6$ fetal liver cells from 15.5dpc $Bmp7^{\Delta/\Delta}$ embryos along with fetal liver cells from 15.5dpc $Bmp7^{+/\Delta}$ control embryos were injected into the tail-vein of the wt irradiated (1,038 rads) CD45.1 mice. At the age of 7-8 weeks mice were sacrificed and

reconstitution was monitored by flow cytometric analysis of peripheral blood using CD45.1/CD45.2 to gate specifically on fetal liver derived cells.

RT-PCR

RNA was extracted from wild-type thymus, bone marrow, liver and kidney and sorted subpopulations using Trizol and concentrations were determined by spectrophotometry. For each fraction 2μg RNA was reverse transcribed into cDNA with superscript II (Invitrogen). RT-PCR was done with in a solution containing 1.2μL $MgCl_2$, 4.0μL mixture containing dNTPs, 1.0μL primer mix, 0.5μL Taq polymerase (Invitrogen). Cycling conditions were as follows: 94°C for 5min, then 35 cycles of 94°C for 20sec, 60°C for 30sec and 72°C for 90 sec. For primer sequences see supplemental methods.

Statistical Analysis

Student's t-test was used for statistical analysis. Results with a P value of less than 0.05 were considered significant.

RESULTS

Multiple components of the Bmp signaling pathway are expressed in primary hematopoietic organs

To be able to address the potential role of Bmp signaling for lymphopoeisis we first assessed the expression of various Bmp and Bmp antagonists in primary lymphoid organs. RT-PCR analysis showed that *Bmp2*, *Bmp4*, *Bmp5*, *Bmp6*, and *Bmp7* were all expressed in bone marrow whereas *Bmp6* expression was not detectable in adult thymocytes. cDNAs from lung and kidney were used as positive controls for the PCR (Fig 1A). Similarly, expression of several Bmp antagonists was found both in thymus and bone marrow, namely *Gremlin*, *Twsg1*, and *Chordin*. *Noggin* expression was restricted to the bone marrow and was not detected in the thymus and (Fig. 1A). The analysis of FACS purified thymocyte sub-populations by RT-PCR revealed that both *Bmp7* and *Twsg1* were expressed in all thymocyte subsets investigated, with somewhat stronger expression in the DN fractions. To confirm these findings we also used lacZ reporter mice to assess gene expression in thymocyte subsets by flow cytometry. We detected expression of *Twsg1* in DN, DP, CD4 and CD8 thymocyte subsets with the relative highest signal in the DN cell compartment (Fig. 1C). FDG-based expression analysis, however, proved not to be sensitive enough to detect *Bmp7* expression in the T cell compartment (Fig. 1D). As expected no expression could be detected for *Bmp4* and *Gremlin* (not shown).

The expression of Bmp/Bmp antagonists also in thymocytes suggests that Bmp signaling might be actively regulating thymopoiesis.

Compartmentalisation of **Bmp/BMP antagonist** *expression in the adult thymus*

To reveal the spatial distribution of the various Bmp/Bmp antagonists within the adult thymus we analyzed lacZ reporter mice for *Bmp4*, *Bmp7*, *Gremlin*, and *Twsg1*. Double staining was performed for LacZ and DEC-205 (CD205) or cytokeratin 8 (CK8) to distinguish between thymic cortex and medulla respectively (Fig. 2). The topology of expression was clearly different for every gene and was typically seen both in the medulla and the cortex. A more systematic analysis of lacZ expression in relation to cell surface markers was performed, which is summarized in Fig. 3 and 4. *Bmp4* was mainly expressed in the vessels of the cortical region as well as in the sub-capsular region of the thymus (Fig. 3A-D) but not in the medullary area where single-positive CD4 or CD8 T cells reside. *Bmp4* does not colocalize with $CD25^+$ thymocytes in the cortical region (Fig. 3A, B) but was observed in dendritic cells and cortical thymic epithelial cells (Fig. 3C, D). *Bmp7* was expressed both in the cortex and in the medulla. Expression was seen in some $CD25^+$ cells (Fig. 3F) but not in $CD3^+$ T cells (Fig. 3E), Furthermore, some dendritic cells and some $CK8^+$ cortical thymic epithelial cells expressed *Bmp7* (Fig. 3G, H). *Twsg1* is abundantly expressed in both cortex and medulla of the adult thymus. It is seen in a few $CD3^+$ cells in the medulla (Fig. 4A) as well as in $CD25^+$ progenitor cells in the cortex (Fig. 4B). Dendritic cells and cortical thymic epithelial cells (Fig. 4C-D) also express *Twsg1*. *Gremlin* is also expressed in the cortex and medulla of the adult thymus. Expression was observed in dendritic cells and few cortical thymic epithelial cells, but not in T cells or their progenitors (Fig. 4E-F).

Bmp/Bmp antagonist expression in the bone marrow

To complement the expression data in thymocytes we performed flow cytometry-based lacZ-expression analysis also on isolated bone marrow cells. We used Ly6C

and CD31 to distinguish blast-like cells, lymphoid cells, myeloid cells, erythrocytes, granulocytes, and monocytes [39]. Bone marrow cells from *Bmp7-lacZ* mice showed weak FDG staining in a subset of lymphoid cells (Fig. 5B). No significant expression of *Bmp4* was observed (Fig. 5A). *Twsg1* expression was detected in nearly all subsets with highest expression in lymphoid cells, monocytes, and granulocytes (Fig. 5C). *Gremlin* expression was detected in erythroid cells, granulocytes, and monocytes. Expression in cells of lymphoid origin varied and was not significant (Fig. 5D). Thus, the expression of various Bmp/Bmp antagonists in different hematopoietic cell subsets strongly suggests that Bmp signaling plays an active role in hematopoiesis.
A more detailed analysis on expression in various B-cell subsets revealed as expected no expression for *Bmp4* but also no clear expression for *Bmp7*. In contrast, expression of *Twsg1* was dynamic, with peak expression seen in pro B-cells. Some *Twsg1* expression was also visible in the pre-pro subset and mature B-cells (Fig. 5B). The expression of *Gremlin* was quite variable and significant expression was only observed in mature B-cells (Fig. 5B).

Steady state lymphopoiesis is unperturbed in Bmp7$^{fl/\Delta}$vav::iCre mice

Subsequently, we focused our analysis on two members of the Bmp signaling family, *Bmp7* and *Twsg1*, and asked whether their deletion from all hematopoietic cells would affect steady-state lymphopoiesis *in vivo*. For this we generated mice lacking *Bmp7* in all hematopoietic cells (Bmp7cKO) by crossing a conditional *Bmp7* allele [38] to the vav::iCre deleter line [40]. Bmp7cKO animals had normal weight and appeared healthy. Analysis of 4-6 weeks old mice revealed that deletion of *Bmp7* had no apparent effects on thymocyte development. The numbers and percentages of progenitor DN, DP, CD4 SP and CD8 SP thymocyte subsets were not significantly altered when

compared to littermate control mice (Fig. 6A). Analysis of the BM from 6-8 weeks mice showed that the cell counts of blast-like cells, erythroid cells, granulocytes, lymphocytes, monocytes, and myeloid cells were all normal when compared to control mice (Fig. 6B). To extend our studies we next transplanted fetal liver cells from 15.5dpc Bmp7-null or control embryos in lethal irradiated, allotypically marked recipient mice. *Bmp7*-deficient mice, obtained by germline deletion of the $Bmp7^{fl/fl}$-allele ($Bmp7^{\Delta/\Delta}$, [38]) survive beyond the embryonic stage E15.5. Transfer of Bmp7-null cells resulted in >80% reconstitution of recipient's immune system by 7-8 weeks. Absence of *Bmp7* did not result in any apparent defects of thymocyte subsets. Similarly, analysis of BM cells revealed unperturbed populations of blast cells, erythroid cells, granulocytes, lymphocytes, monocytes and myeloid cells (Fig. S1). Taken together, the results obtained by a lineage specific deletion of *Bmp7* and by the transplantation of fetal *Bmp7*-null progenitors reveals that Bmp7 secretion from any hematopoietic subset is dispensable for lymphopoiesis *per se*.

Steady state lymphopoiesis is unperturbed in Twsg1$^{fl/KO}$vav::iCre mice

Twsg1, similar to *Bmp7*, is expressed both by thymocytes and by thymic stroma cells. *In vitro* analyses have shown that Twsg1 regulates thymocyte development at the DN and DN to DP transitional stage [26, 27]. As *Twsg1*-deficient mice display defects in lymphopoiesis [41] we sought to determine whether these defects are due to lack of Twsg1 in the lymphoycte precursor cells or the respective stroma cells. For this we generated mice lacking *Twsg1* in all hematopoietic cells (Twsg1cKO) by crossing a conditional $Twsg1^{fl/fl}$ allele [37] to the vav::iCre deleter line [40]. Twsg1cKO animals appeared normal and remained healthy for up to 10-12 months. Analysis of thymocyte subsets from 4-6 weeks old Twsg1cKO or control mice by flow cytometry revealed

that absence of *Twsg1* in the hematopoietic cell subset did not affect thymocyte development. The DN, DP, as well as CD4 SP and CD8 SP thymocyte subsets were not altered (Fig. 7A). This indicated that lack of stroma-derived rather than thymocyte-derived Twsg1 is responsible for the lymphoid defects observed in *Twsg1*-deficient mice [41]. Similarly, analysis of bone marrow cells from 6-8 weeks mice showed that the relative number of blast-like cells, erythroid cells, granulocytes, lymphocytes, monocytes, and myeloid cells was not altered (Fig. 7B). Moreover, analysis of developing B cells showed that pre-pro ($B220^{+}/CD19^{-}/cKIT^{-}/CD25^{-}/IgM^{-}$), pro ($B220^{+}/CD19^{+}/cKIT^{+}/CD25^{+}/IgM^{-}$), pre ($B220^{+}/CD19^{+}/cKIT^{-}/CD25^{+}/IgM^{+}$), immature ($B220^{+}/CD19^{+}/cKIT^{-}/CD25^{-}/IgM^{+}$) cells remained unaltered (Fig. 7C). These data indicate that despite its abundant expression in thymocytes and B-lymphocyte precursors, lymphopoiesis is not critically dependent on *Twsg1*-expression in the hematopoietic compartment.

Discussion

The importance of Bmp signaling for the development and homeostasis of thymus and bone marrow is clearly documented [6, 24, 26, 28, 41]. Several different Bmps (Bmp2, Bmp4, Bmp5, Bmp6 and Bmp7) and Bmp antagonists (Noggin, Gremlin, Twsg1, and Chordin) are expressed both in thymus and the bone marrow. Using LacZ reporter mice for several Bmp/Bmp antagonists in combination with histochemistry we have mapped expression of *Bmp4*, *Bmp7*, *Gremlin*, and *Twsg1* in the thymus and developing lymphocytes revealing highly unique expression pattern for every gene analysed. *Bmp4* was mainly expressed along vessels of the cortical region as well as in the sub-capsular region of the thymus but not in the medullary area where single positive T cells reside. Quite in contrast *Bmp7* expression was observed both in the cortex and in the medulla, in epithelial cells, dendritic cells, as well as some developing lymphocytes. Both *Twsg1* and *Gremlin* were abundantly expressed in cortex and medulla. Expression of both molecules was noted in epithelial cells and dendritic cells. As expected, *Twsg1* was also found in developing thymocytes. It is noteworthy that *Twsg1* expression in the cortex was not uniform but confined to distinct areas. This could indicate a hitherto unrecognized functional compartmentalization of the thymic cortex. Twsg1 might also differentially affect CD4 and CD8 T-cells. We frequently observed $CD3^+$ T-cells in close vicinity to *Twsg1*+ medullary stroma cells we never saw this for $CD8^+$ T-cells. Though we mapped expression for only a few Bmp/Bmp antagonists, this limited survey was sufficient to indicate an unexpected complexity of Bmp signaling in the adult thymus where multiple Bmp signaling networks regulate thymus homeostasis and thymocyte development. With respect to the thymocytes, Bmp/Bmp antagonist expression

appeared compartmentalised in cortical and medullary areas of the thymus suggesting that Bmp signals might affect all stages of T-cell development.

Relatively little is known about how BMP signals regulate the various aspects of thymocyte differentiation [42]. *In vitro* experiments have established that BMP2/4 negatively affect the transition from the DN2 to the DN3 and from the DN to the DP stage. Addition of exogenous BMP antagonists such as Noggin or Chordin/Twisted Gastrulation reversed these effects [26, 27]. Thus, the balance between BMP/BMP antagonists might regulate T-cell development within the various thymic microenvironments *in vivo*. Thereby, BMP signals could be acting on thymocytes directly or indirectly via the thymic stroma.

One prominent example involving direct signaling of Bmps is their regulation of *Id* (Inhibitor of differentiation) gene expression [43]. Ids inhibit the E-proteins E47 and E12, two important regulators of thymocyte development. Ids have also been mapped downstream of TCR signaling [44]. Overexpression of a dominant negative form of Id3 in human T lineage precursor cells blocks early T cell development [45]. In addition, the observation that the BMP antagonist Twsg1 is upregulated following preTCR engagement [26] might also be regarded as evidence for a direct effect of Bmp signals on developing thymocytes. As the degradation of E-proteins is regulated by Notch signaling [46], Id/E protein activity might be a major inegration platform of various major signaling pathways.

Futhermore, Bmps appear to be important regulators of the thymic stroma *per se* [6] acting mainly indirectly by engaging several cellular signaling pathways. This can be achieved in part through regulating *FoxN1* gene expression as was shown for the embryonic thymic stroma [47]. Whether this mechanism also operates in the adult thymus needs to be shown. Comparisons with other organs would indicate that Bmp

signaling can interact with all major developmental signaling pathways such as Notch, wnt/b-catenin, Hh and FGF. All these pathways have established roles in thymus and thymocyte development on their own[6-9], but comparatively little is known on reciprocal interactions between these pathways. Interestingly, Bmp4 reportedly can modulate FGF7/FGF10 [24] in the thymic stroma. In addition, BMP signals might also interfere with cytokine signaling, as shown for Bmp4, which negatively regulates IL-7-mediated development of thymic progenitor cells [28].

With respect to Bmp/Bmp antagonist expression in developing thymocytes only *Bmp7* and *Twsg1* were detected. They were expressed in all thymocyte subsets investigated, and for both expression was strongest in DN thymocyte subsets. *Twsg1* expression in DN thymocyte subsets had been reported previously [26, 28]. In this report we show *Twsg1* is also expressed in DP, CD4SP, and CD8SP thymocytes. *Twsg1* can be induced in DP thymocytes in a TCR-dependent manner [26], thus its expression might reflect their. *Twsg1* was also dynamically expressed in various subsets of developing B-cells. Highest expression was observed in pro B-cells, which like the DN2 thymocyte population is known to require cytokine survival signals, most prominantely IL-7 [48, 49]. As Bmp4 can counteract this IL-7-induced proliferation and differentiation [28], *Twsg1* expression in these subsets could serve to overcome a Bmp-mediated proliferative block, similarly to its role in regulating the DN to DP transition in thymocytes [26]. *Twsg1* was also expressed in all other hematopoietic subsets analyzed indicating a function for Bmp signaling also for their development.

The expression of *Bmp7*, which was strongest in the DN3/DN4 thymocyte subsets, could not be confirmed in thymocytes or bone marrow cells using the *Bmp7*-lacZ reporter mouse [34]. Possible explanations for this might be low expression and/or insufficient sensitivity of the lacZ reporter line caused by the incidental disruption of

a lymphocyte specific enhancer when inserting the lacZ gene into the Bmp7 locus or alternatively, by the silencing of promoter structures instigated by the presence of the lacZ gene.

In the BM Bmp signaling has mainly been associated with a role in regulating the stem cell-niche synapse [50]. The expression of *Bmp7*, *Twsg1*, and *Gremlin* in developing hematopoietic cells would therefore suggest that Bmp signaling regulates the maturation of all hematopoietic cell lineages. This might be direct by regulating their development or indirect by providing signals to their niche or to the close cellular environment.

In the thymus developing lymphocytes need to interact with thymic stroma cells for a functional organ to develop [29]. Thus, the necessity for reciprocal crosstalk is well established in this organ. Thymic epithelial cells are constantly regenerated from a pool of stem/progenitor cells in the adult thymus making the thymus a much more dynamic structure than previously assumed [51]. It has been shown that Bmp signals maintain the expression of the critical *FoxN1* gene in embryonic thymic stroma [47]. Currently, it is not known whether in the adult thymus *FoxN1* expression is also regulated by Bmp signals, and if yes, what is the identity and source of this Bmp signal. It will be of particular interest to establish whether lymphocyte-derived derived Bmp signals participate in this process.

Bmp7-deficient mice are not viable [38, 52, 53] and the *in vivo* requirements for *Twsg1* are background dependent. Whereas *Twsg1*-deficient mice are viable and show impaired lymphocyte development on some genetic backgrounds [41], about 44% of *Twsg1* null mutants on the C57Bl6 background die *in utero* and display craniofacial malformations of variable severity ([37], and own observations). Conditional deletion of

Bmp7 and *Twsg1* in all hematopoietic cells using the vav::iCre resulted in viable and healthy mice with normal cellularity of the lymphoid organs. We also analyzed 10-12 months old Bmp7cKO and Twsg1coKO mice to assess potential late-onset effects, but still did not observe any alterations to the hematopoietic compartments (data not shown). This indicated that bone-marrow stroma derived Twsg1 rather than hematopoietic cell derived Twsg1 might be responsible for the lymphoid defects observed in *Twsg1* null mice [41].

To extend these results we transplanted *Bmp7*-deficient fetal liver cells into lethally irradiated wild type recipients, which revealed that *Bmp7* was even dispensible for the development of embryonic hematopoietic stem cells. Taken together, these data suggest that although *Twsg1* and *Bmp7*-are dynamically expressed in lymphoid and some myeloid cells, they appear to be redundant for steady-state lymphoid and myeloid development. How can this be reconciled with published *in vitro* data where Twsg1 has been shown to counteract Bmp2/4, which are both negative regulators of lymphopoiesis [26-28]? One obvious possibility is their compensation from other cellular sources or functional redundancy. Stroma cells also produce Twsg1 and Bmp7. This compartment is seemingly the critical contributor of Twsg1 *in vivo* given the overall undisturbed lymphopoiesis in Tswg1$^{fl/fl}$vav::iCre animals. Since *Bmp7*-deficient animals die in late gestation or shortly after birth [38] we have no information on its role for T- or B-cell development. Some preliminary analysis of fetal *Bmp7*-deficient thymi indicates altered thymocyte development in the absence of Bmp7 (O. Passa and D. Graf, unpublished). Compensation by other Bmps, such as Bmp5 or Bmp6 that belong to the same Bmp subgroup (glass-bottom-boat orthologues) is also a possibility. In contrast, a functional compensation by Bmp2/4 (decapentaplegic orthologues) is less likely, as Bmp7 itself did not recapitulate the inhibitory effects of

Bmp2/4 on thymocyte proliferation and differentiation [26]. Why would developing lymphocytes express Bmp/Bmp antagonists if their function appears to be redundant? Our analysis of *Twsg1*- and *Bmp7*-deficiency was restricted to steady state lymphoid development. Even if lymphoid development *per se* is not affected, the functional properties of the mature B- and T-cells might be altered in their absence. For instance, B-cell derived *Twsg1* is not required for B-cell development *per se*, but is involved in regulating T-cell independent plasma B-cells production and function [54]. Thus, Bmp signaling might fine-tune lymphocyte development, which could be achieved by altering cytokine or TCR/BCR signaling thresholds. In consequence, absence of these molecules might affect repertoire selection or recruiting into different functional subsets. On the other hand, thymic epithelial cells might also be targets of lymphocyte derived BMP signals. Both cortical and medullary epithelial cells are continuously replenished from epithelial stem/precursor cells [51]. It is thus feasible that lymphocyte-derived BMP signals are part of the thymocyte-thymic stroma crosstalk and contribute to the maintenance of a functional thymus.

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FIGURE LEGENDS

Figure 1. Expression of Bmp/Bmp antagonists in primary lymphoid organs. (A) RT-PCR analysis of Bmps, Bmp antagonists in the thymus and bone marrow. Lung and kidney served as positive controls. **(B)** *Bmp7* and *Twsg1* expression on FACS sorted DN2-4, DP, SP CD4 and SP CD8 thymocyte populations. **(C-D)** FDG based quantitative analysis of lacZ gene reporter activity by FACS on thymocyte subpopulations from *Twsg1-lacZ* (C), *Bmp7*-lacZ (D) and wt control mice showing significant levels of expression for *Twsg1* in the SP CD4, SP CD8, DP and DN compartments in the thymus (n=9 mice per group), while no significant differences were detected for *Bmp7* (n=5 mice per group). MFI, Mean Fluorescence Intensity, * $p<0.05$.

Figure 2. Bmp/Bmp antagonist expression in cortex and medulla of the thymus. (A-D) X-gal based detection of lacZ gene reporter activity for *Bmp4* (A), *Bmp7* (B), *Twsg1* (C), *Gremlin* (D)(blue) on thymus sections combined with immunohistochemical staining for DEC-205 (CD205, NLDC; brown) to indicate cortical and medullary areas. c, cortex; m, medulla; sc, subcapsular zone. (size bar 50µm).

Figure 3. Identity of *Bmp4* and *Bmp7* expressing cells in the thymus. X-gal based detection of lacZ gene reporter activity for *Bmp4* (A-D) and *Bmp7* (E-H) (blue) on thymus sections combined with immunohistochemical staining for for CD3 (A, E), CD25 (B, F), DEC-205 (C, G), Cytokeratin8 (CK8) (D, H) (red or brown). c, cortex; m, medulla; sc, subcapsular zone. (size bar 50µm).

Figure 4. Identity of *Twsg1* and *Gremlin* expressing cells in the thymus. X-gal based detection of lacZ gene reporter activity for *Twsg1* (A-D) and *Gremlin* (E-H) (blue) on thymus sections combined with immunohistochemical staining for for CD3 (A, E), CD25 (B, F), DEC-205 (C, G), CK8 (D, H) (red or brown). c, cortex; m, medulla; sc, subcapsular zone. (size bar 50μm).

Figure 5. *Bmp7*, *Twsg1* and *Gremlin* are expressed in hematopoietic cells in the BM. (A) FDG based quantitative analysis of lacZ gene reporter activity of *Bmp4, Bmp7, Twsg1,* and *Gremlin* by FACS on bone marrow subpopulations identified by Ly6C/CD31 staining[55].(A) or B-cell subpopulations identified by B220/CD19/cKIT/CD25[2] (B). (A) Significant expression for *Bmp7* was observed in lymphoid cells (n=4 mice per group), for *Twsg1* in erythroid cells, granulocytes, lymphoid, and myeloid cells as well as monocytes (n=5 mice per group), for *Gremlin* in the erythroid cells, granulocytes and monocytes (n=4 mice per group), while no expression of Bmp4 was detected (n=4 mice per group). **(B)** Significant expression for *Twsg1* was observed in pre-pro ($B220^+/cKIT^-/CD19^-$), pro $B220^+/CD19^+/cKIT^+/CD25^+$) and B cells ($B220^+/CD19^+/cKIT^-/CD25^-$) while *Gremlin* expression was limited to B cells ($B220^+/CD19^+/cKIT^-/CD25^-$). Bl, Blasting cells; L, Lymphocytes; E, Erythrocytes; My, Myeloid precursor cells; G, Granulocytes; Mo, Monocytes; MFI, Mean Fluorescence Intensity, * $p<0.05$.

Figure 6. Normal lineage distribution in Bmp7cKO mice in thymus and BM. Analysis of thymocyte (A) and bone marrow (B) subpopulations and graphical representation of cell numbers from Bmp7cKO and littermate control mice. (n=4 mice

per group). The results are representative for more than 3 independent experiments. (A) Thymocyte populations were detected by CD4/CD8 staining and gated as DN, DP, CD4SP, CD8SP subpopulations. (B) Bone marrow populations were detected by CD31/Ly6C staining[55]. Percentages and total numbers of the various subsets were unaltered (n=4 mice per group). The results are representative for more than 3 independent experiments. Bl, Blasting cells; L, Lymphocytes; E, Erythrocytes; My, Myeloid precursor cells; G, Granulocytes; Mo, Monocytes

Figure 7. Normal lineage distribution in Twsg1cKO mice in thymus and BM. Analysis of thymocyte (A) and bone marrow (B) subpopulations and graphical representation of cell numbers from Twsg1cKO and littermate control mice. (n=3 mice per group). The results are representative for more than 3 independent experiments. (A) Thymocyte populations were detected by CD4/CD8 staining and gated as DN, DP, CD4SP, CD8SP subpopulations. (B) Bone marrow populations were detected by CD31/Ly6C staining[55]. Percentages and cell numbers of the various subsets were unaltered (n=4 mice per group). The results are representative for more than 3 independent experiments. (C) B-cell subpopulations identified by B220/CD19/cKIT/CD25/IgM staining[2]. Percentages and cell numbers of the various subsets were unaltered (n=3 mice per group). Bl, Blasting cells; L, Lymphocytes; E, Erythrocytes; My, Myeloid precursor cells; G, Granulocytes; Mo, Monocytes

Figure S1. Normal lineage distribution of *Bmp7*$^{\Delta/\Delta}$ cells after BM transplantation of E15.5 fetal liver *Bmp7*$^{\Delta/\Delta}$ cells. Analysis of thymocyte (A) and bone marrow (B) subpopulations and graphical representation of cell numbers from Bmp7cKO and littermate control mice. (n=4 mice per group). (A) Thymocyte populations were detected by CD4/CD8 staining and gated as DN, DP, CD4SP, CD8SP subpopulations. (B) Bone marrow populations were detected by CD31/Ly6C staining and gated as described[50]. Percentages and cell numbers of the various subsets were unaltered (n=4 mice per group). Bl, Blasting cells; L, Lymphocytes; E, Erythrocytes; My, Myeloid precursor cells; G, Granulocytes; Mo, Monocytes

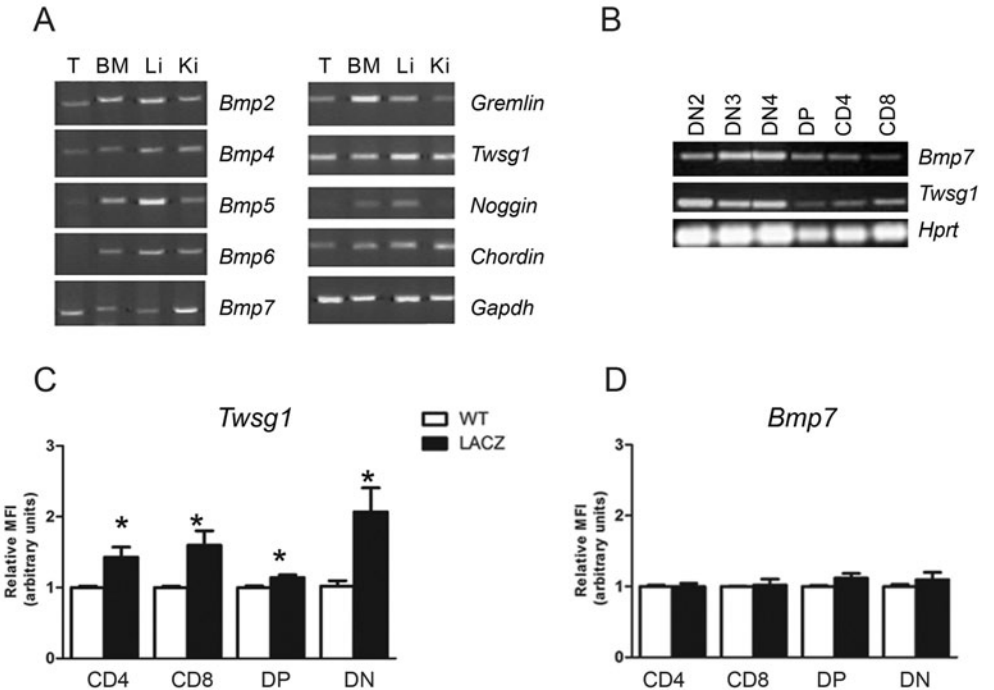

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Figure 1

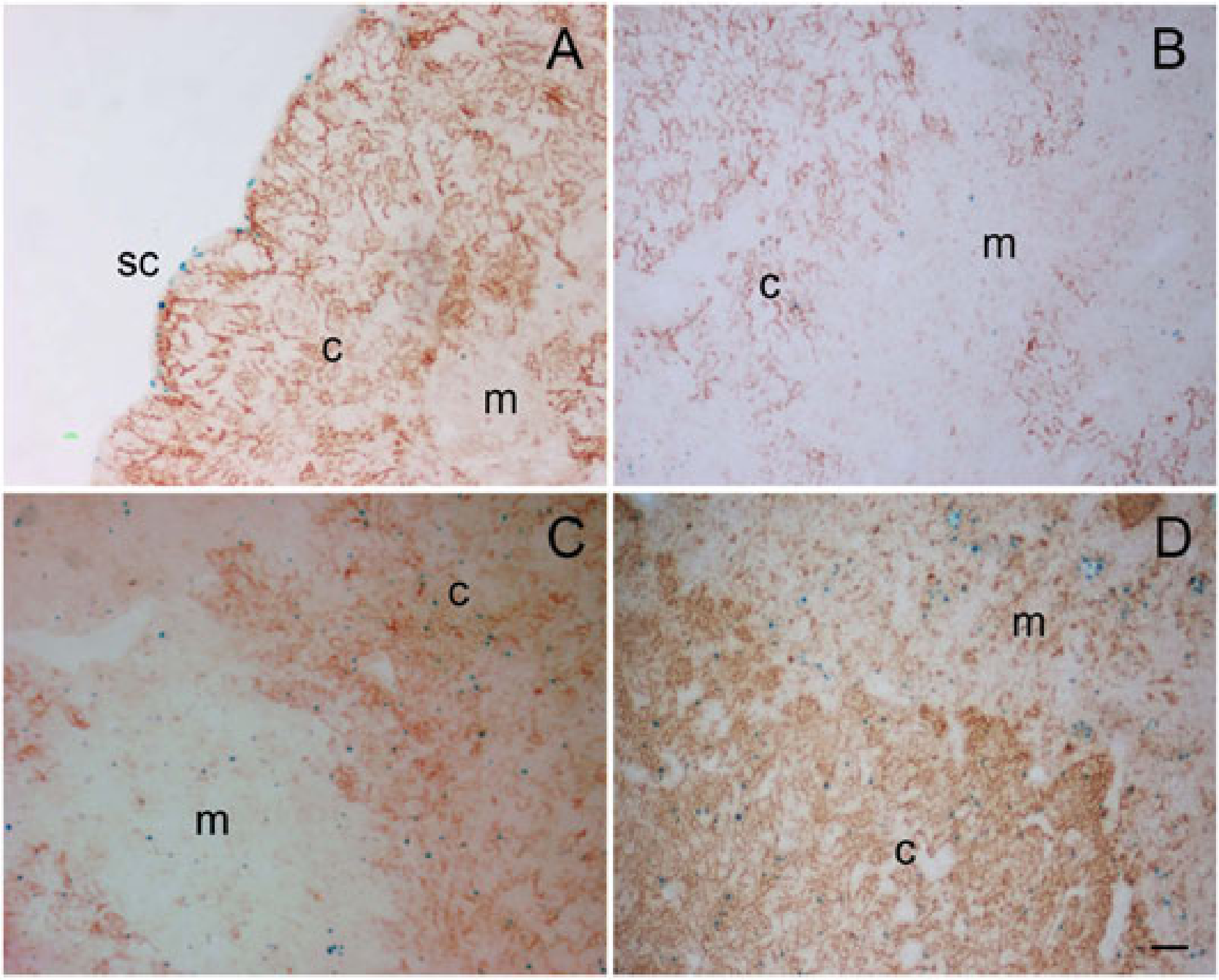

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Figure 2

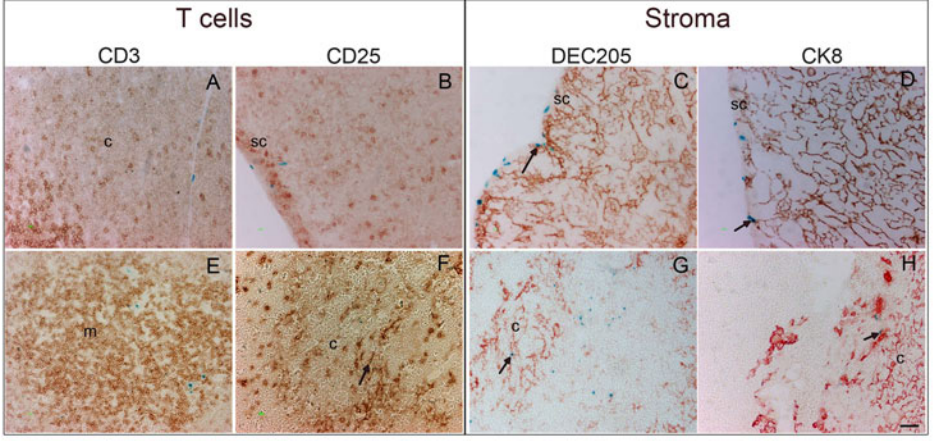

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Figure 3

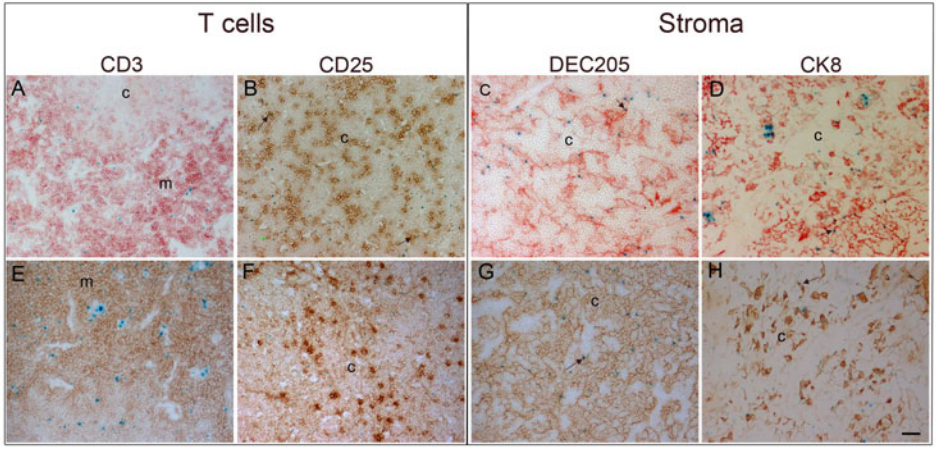

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Figure 4

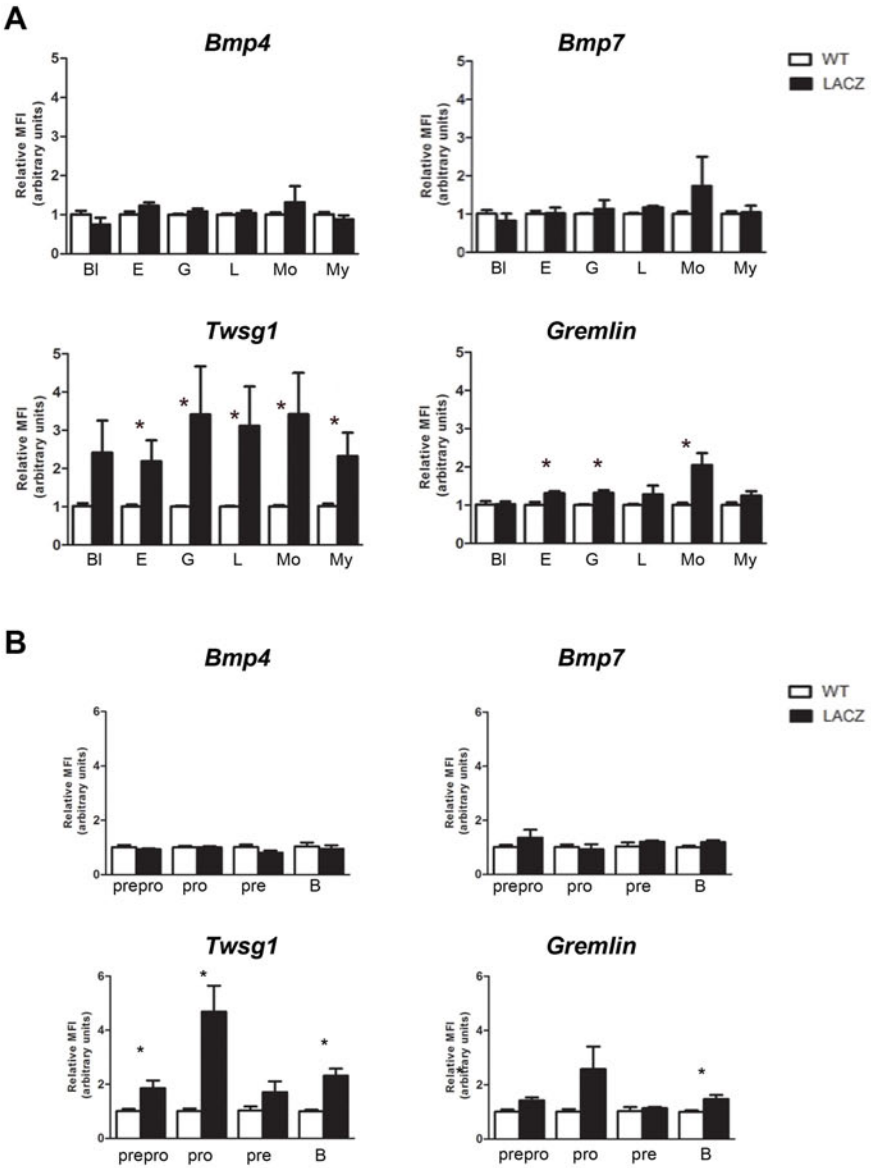

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Figure 5

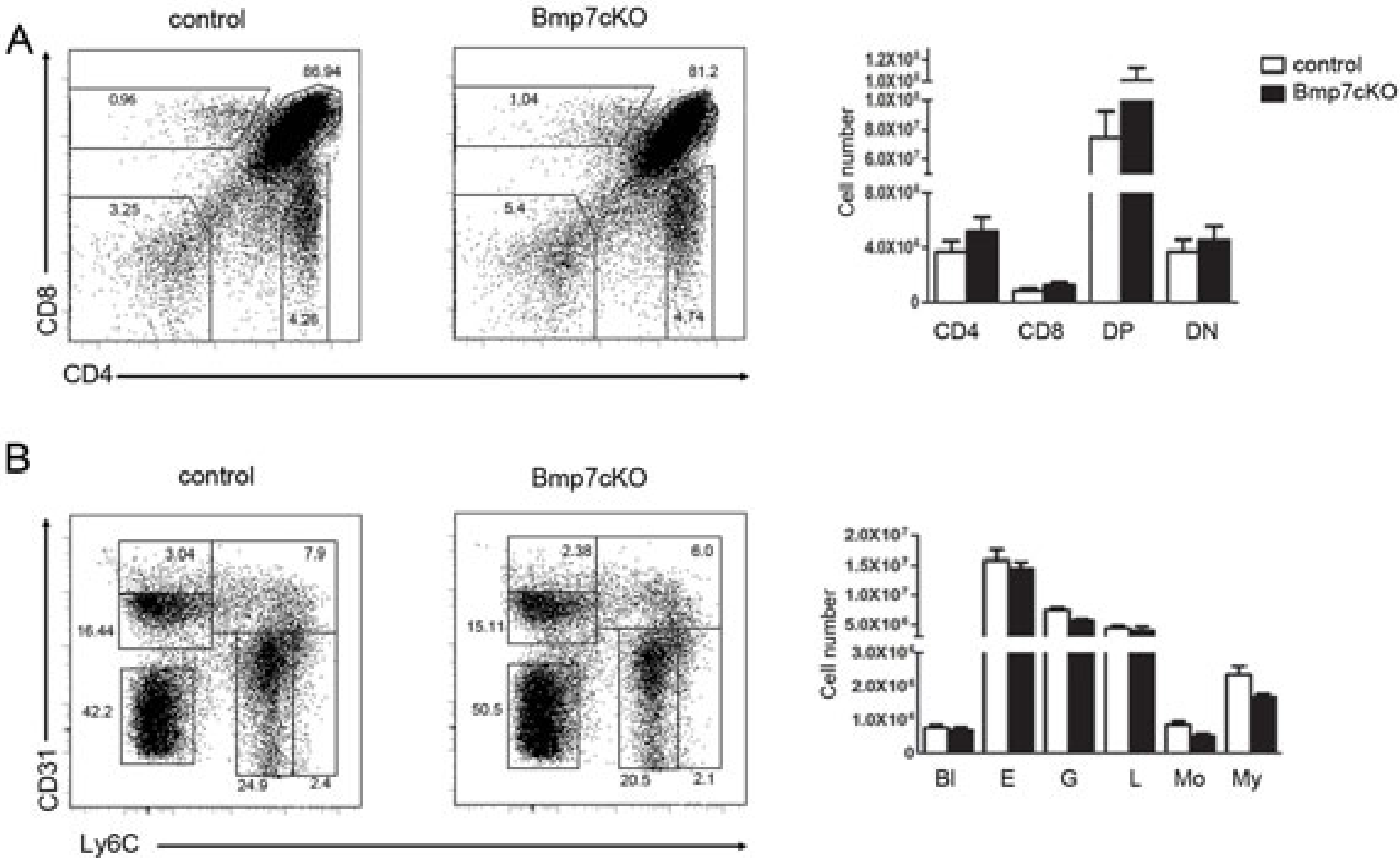

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Figure 6

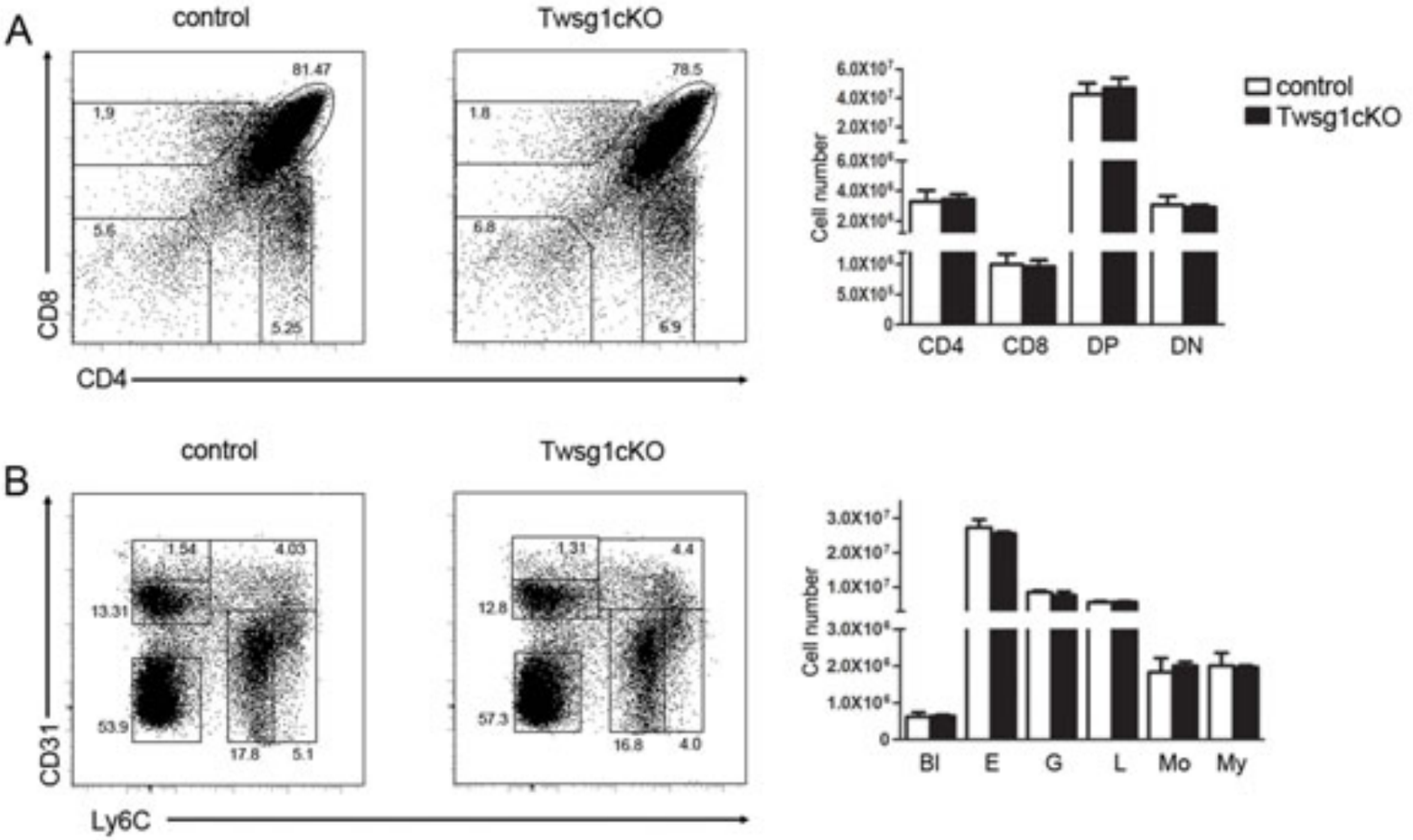

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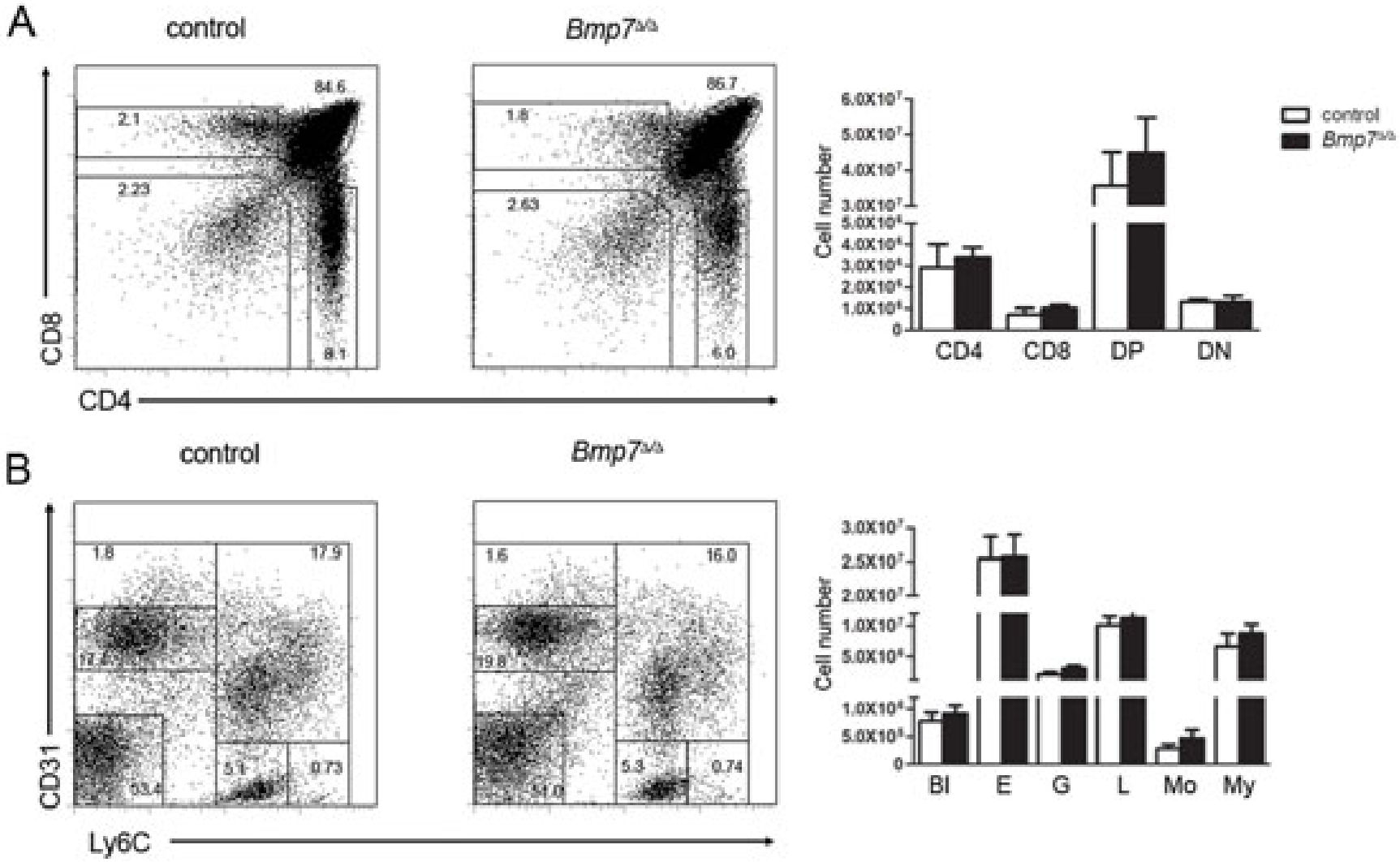
Figure 7



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Supplementary Figure 1